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Antidepressant-like effect of losartan involves TRKB transactivation from angiotensin receptor type 2 (AGTR2) and recruitment of FYN

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ABSTRACT

The renin-angiotensin system (RAS) is associated with peripheral fluid homeostasis and cardiovascular function, but recent evidence also suggests a functional role in the brain. RAS regulates physiological and behavioral parameters related to the stress response, including depressive symptoms. Apparently, RAS can modulate levels of brain-derived neurotrophic factor (BDNF) and TRKB, which are important in the neurobiology of depression and antidepressant action. However, the interaction between the BDNF/TRKB system and RAS in depression has not been investigated before. Accordingly, in the forced swimming test, we observed an antidepressant-like effect of systemic losartan but not with captopril or enalapril treatment. Moreover, infusion of losartan into the ventral hippocampus (vHC) and prelimbic prefrontal cortex (PL) mimicked the consequences of systemically injected losartan, whereas K252a (a blocker of TRK) infused into these brain areas impaired such effect. PD123319, an antagonist of AT2 receptor (AGTR2), also prevented the systemic losartan effect when infused into PL but not into vHC. Cultured cortical cells of rat embryos revealed that angiotensin II (ANG2), possibly through AGTR2, increased the surface levels of TRKB and its coupling to FYN, a SRC family kinase. Higher *Agtr2* levels in cortical cells were reduced after stimulation with glutamate, and only under this condition an interaction between losartan and ANG2 was achieved. TRKB/AGTR2 heterodimers were also observed, in MG87 cells GFP-tagged AGTR2 co-immunoprecipitated with TRKB. Therefore, the antidepressant-like effect of losartan is proposed to occur through a shift of ANG2 towards AGTR2, followed by coupling of TRK/FYN and putative TRKB transactivation. Thus, the blockade of AGTR1 has therapeutic potential as a novel antidepressant therapy.

1. Introduction

The functional role of the renin-angiotensin system (RAS) has historically been implicated in cardiovascular and fluid homeostasis. The precursor molecule angiotensinogen is cleaved by renin into angiotensin I, which is then converted into angiotensin II (ANG2) by angiotensin-converting enzyme (ACE) (Wright and Harding, 2011). The primary actions of ANG2 are mediated by angiotensin II receptors type 1 (AGTR1) and 2 (AGTR2) (Wright and Harding, 2011).

Other reports, however, have indicated that all components of RAS are produced within the central nervous system (CNS) (Saavedra, 2005). Thus, AGTR1 and AGTR2 in circumventricular organs and in cerebrovascular endothelial cells may respond to circulating ANG2 of peripheral origin, whereas neural receptors inside the blood brain barrier respond to RAS of brain origin (Saavedra, 2005). AGTR1 and AGTR2 are expressed inside blood brain barrier structures such as the hippocampus and frontal cortex (Bunnemann et al., 1992; Tsutsumi and Saavedra, 1991), which are both considered crucial limbic structures associated with the neurobiology of depression (Krishnan and Nestler, 2008).

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In fact, several pieces of evidence suggest that ANG2 is a hormone regulator of peripheral and central physiological changes regarding stress exposure, including behavioral consequences. For instance, both acute and chronic stress increased ANG2 and AGTR1 expression levels in the hypothalamic-pituitary-adrenal axis (HPA axis) (Castren and Saavedra, 1988; Saavedra et al., 2004; Yang et al., 1993). Moreover, candesartan (an AGTR1 antagonist) treatment prevented the stress effect of increasing pituitary adrenocorticotropic and corticosterone hormone levels (Armando, 2001), and treatment with ACE inhibitors (ACEi) or AGTR1 antagonists reversed or prevented animal behavioral responses to stress (Gard et al., 1999; Giardina and Ebert, 1989; Martin et al., 1990a, 1990b; Ping et al., 2014; Vijayapandi and Nagappa, 2005). Similarly, animals lacking angiotensinogen showed an antidepressant-like phenotype (Okuyama et al., 1999).

The neurotrophin brain-derived neurotrophic factor (BDNF) is found mostly in the central nervous system and is important for neural plasticity, including synapse formation, neuronal differentiation, and growth (Park and Poo, 2013). The functional role of BDNF and its receptor (tropomyosin-related kinase B receptor, TRKB) has been linked to the pathophysiology of psychiatric disorders (such as depression) and with the mechanism of action of antidepressant drugs (Castrén, 2014). RAS may modulate BDNF and TRKB brain levels. For instance, candesartan treatment prevented both infarct volume and neurological deficits in animals submitted to middle cerebral artery occlusion, and increased TRKB protein and mRNA levels in the brain (Krikov et al., 2008). In addition, telmisartan (an AGTR1 antagonist) chronic treatment prevented retinal damage and decrease of BDNF levels in a diabetic animal model (Ola et al., 2013). Valsartan, another AGTR1 antagonist, counteracted the consequences of stress on depressive and anxiogenic-like behavior and on BDNF levels in the hippocampus and frontal cortex (Ping et al., 2014). Moreover, some case reports describe relief of depressive symptoms in hypertensive patients treated with the ACEi captopril (Deicken, 1986; Germain and Chouinard, 1988; Zubenko and Nixon, 1984).

Despite scarce evidence, it is plausible that drugs acting on the RAS promote antidepressant-like effects. However, such properties have not been linked to modulation of the BDNF/TRKB system. In the present work, we investigated the behavioral effects of the AGTR1 antagonist losartan and ACEi in a model predictive of antidepressant-like effect (*i.e.*, forced swimming test) and the requirement of BDNF/TRKB for such effect. Since AGTR1 activation is related to brain injury (Saavedra, 2012) and activation of AGTR2 induces neuroprotective outcomes, especially when AGTR1 is blocked (Mogi and Horiuchi, 2013; Zhao et al., 2005), we hypothesized that activation of AGTR2 mediates the antidepressant-like effects of losartan. We also performed a series of *in vitro* analysis to provide a mechanistic insight to the behavioral data.

2. Material and methods

2.1. Animals

Male Wistar rats (250–350 g) and female C57BL6/j mice (20–25 g), heterozygous to BDNF expression or WT littermates were used in behavioral studies. The rats were housed in pairs while mice were group-housed (4–6/cage) in temperature-controlled room (24 ± 1 °C), under standard laboratory conditions with access to food and water *ad libitum* and a 12-h light/12-h dark cycle (light on at 6:30 a.m.). *In vivo* experiments were conducted with approval of the local Ethical Committee (protocol 147/2017 for University of São Paulo and ESAVI/10300/04.10.07/2016 for University of Helsinki), which are in accordance with the Brazilian Council for the Control of Animals under Experiment (CONCEA), and the European Union and ARRIVE guidelines (Kilkenny et al., 2010) for the care and use of laboratory animals. All licenses comply with international laws and policies.

2.2. Cell culture

Mouse fibroblasts stably overexpressing full-length TRKB (MG87.TRKB) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine, and 400 mg/ml G418). Cell lines were maintained at 5% CO_2 and 37 °C until reaching 70% confluence for experiments. For primary neuronal cultures, cortices from E18 rat embryos were dissected and tissue was dissociated with papain solution in PBS for 10 min at 37 °C. Cells were suspended in DMEM medium containing Ca^{2+}/Mg^{2+}-free HBBS, 1 mM sodium pyruvate, 10 mM HEPES (pH = 7.2) and DNAse, and plated onto poly-*l*-lysine-coated 24-well or 96-well plates at a cell density of 125 000 cells/cm^2. Primary neurons were maintained in Neurobasal medium (supplemented with 2% B27, 1% penicillin/streptomycin and 1% L-glutamine) and supplemented with fresh medium every third day.

2.3. Drug treatments

Losartan potassium (losartan; AGTR1 antagonist; Pharmanostra, Brazil) was administered intraperitoneally (ip) in rats (10, 30, and 45 mg/kg), intracerebrally (0.1, 1, and 10 nmol/site), in cell cultures (10μM; Tocris #3798, USA) and ip in mice at 45 mg/kg. Captopril (angiotensin converting enzyme inhibitor - ACEi; 3, 10, and 30 mg/kg; Pharmanostra, Brazil) and enalapril (ACEi; 1, 3, and 10 mg/kg; Pharmanostra, Brazil) were injected ip. K252a (TRK antagonist, Sigma-Aldrich, #K2015, Germany) was used for intracerebral (20mM/site), or in culture (10μM) treatment. PD123319 (PD; AGTR2 antagonist; Tocris, #1361, USA) was used for intracerebral (200μM/site) or culture (200 nM) treatment. ANG2 (10μM; Tocris #1158, USA), BDNF (2.5 ng/ml, Peprotech, USA), NGF (10 ng/mL, Sigma-Aldrich, Germany), TRKB.Fc (200 ng/ml; R&D systems, #688-TK-100, USA), and CGP42112 (CGP; AGTR2 agonist; 2uM; Tocris, #2569, USA) were used only for cell culture treatment. K252a and PD123319 intracerebral doses were chosen based on previous studies (Jiang et al., 2012; Zheng et al., 2008). 2.5% 2,2,2 tribromoethanol (ip, Sigma-Aldrich, #T48402) and subcutaneous local anesthetic lidocaine (PROBEM 3%, 0.2 ml) were used for stereotaxic surgery. Chloral hydrate (0.75 g/kg, ip, Sigma-Aldrich, #C8383, Germany) was used to euthanize animals for perfusion. Subcutaneous banamine (Schering-Plough, 0.25%, 0.1 ml/100 g) and intramuscular oxytetracycline (Pfizer, 20%, 0.1 ml/100 g) were used once for postoperative recovery. Losartan, tribromoethanol, chloral hydrate, and banamine were freshly prepared in saline solution. All other drugs were prepared in 0.1% DMSO in saline.

2.4. Surgery, intracerebral injections, and histology

Surgery and intracerebral drug injections were performed as previously described (Diniz et al., 2016). Briefly, rats were anesthetized with tribromoethanol and fixed in a stereotaxic frame. Stainless steel guide cannulas (0.7 mm OD) aimed at the dorsal hippocampus (dHC; coordinates: AP = −4.0 mm from bregma, L = 2.8 mm, DV = 2.1 mm), ventral hippocampus (vHC; coordinates: AP = −5.0 mm from bregma, L = 5.2 mm, DV = 4.0 mm), or prelimbic ventromedial prefrontal cortex (PL; coordinates: AP = +3.3 mm from bregma, L = 1.9 mm, DV = 2.4 mm; lateral inclination of 22°) were implanted according to the atlas of Paxinos and Watson (1998) and attached to skull bone with stainless steel screws and acrylic cement. A stylet inside the guide cannula prevented obstruction. Five to seven days after surgery, intracerebral

injections were performed with a dental needle (0.3 mm OD) in a volume of 200 nl (mPFC) or 500 nl (dHC or vHC) infused for 1min using a Hamilton microsyringe (Sigma-Aldrich) and infusion pump (KD Scientific) 20min before the test session. After behavioral tests, rats were anesthetized with chloral hydrate and 200 nl of methylene blue was injected through the guide cannula. The brains were removed and injection sites verified. Results from injections outside the targeted area were discarded from statistical analysis. All histological sites of injection were inserted in diagrams (Figs. S2 and S3) according to the atlas of Paxinos and Watson (1998).

2.5. Forced swimming test

The forced swimming test (FST) was performed on rats as follows: animals were placed individually to swim in a Plexiglas cylinder (24 cm diameter by 60 cm height with 28 cm of water at 25 ± 1 °C) for 15min (pretest). Twenty-four hours later, animals were placed again in the cylinder for a 5min swim test session and immobility time was measured (Diniz et al., 2016).

The FST in mice was performed as follows: the animals were placed in 5 L glass beaker cylinders (19 cm diameter, with a 20 cm water column) for 6min. The immobility was assessed in the last 4min of the session (Diniz et al., 2017). For both protocols, water was changed between each test. After swimming, animals were towel-dried and kept in a warmed cage before returning to home cages. The test was videotaped and analyzed by a trained observer blind to treatment.

2.6. Overexpression of GFP-AGTR2

MG87 cells were transfected to express GFP-tagged AGTR2 using lipofectamine (Jiang et al., 2012). Briefly, at a confluence of 70%, the cells were incubated with a mixture of 2.5% lipofectamine 2000 (Thermo Scientific, #11668019) and 5ug/ml of the plasmid in OptiMEM medium. Forty-eight hours after transfection, cells were treated, lysed, and subject to immunoprecipitation as described below.

2.7. Sample collection

For immunoassays, cells were washed with ice-cold PBS and lysed (137 mM NaCl, 20 mM Tris-HCl, 1% NP40, 0.5 mM NaF, 10% glycerol, pH = 7.4; supplemented with protease/phosphatase inhibitor cocktail [Sigma-Aldrich, #P2714; #P0044] and sodium orthovanadate [0.05%, Sigma-Aldrich, #S6508]). Samples were then centrifuged at 10000 × g for 15 min at 4 °C. Supernatant was collected and stored at −80 °C until use. For polymerase chain reaction, the cells were washed with PBS and treated with Qiazol Lysis Reagent™ (Qiagen). Lysate was collected in a clean tube and incubated with chloroform for 3 min at room temperature (RT). After centrifugation at 15200 × g for 10 min at 4 °C, the aqueous phase was mixed with isopropanol for 10 min at RT and centrifuged at 15200 × g for 10 min at 4 °C. The pellet was washed twice with 75% EtOH, once with 100% EtOH, air-dried, and dissolved in 20ul MQ water.

2.8. Reverse transcription-polymerase chain reaction and quantitative polymerase chain reaction

The concentration and purity of each RNA sample were determined using NanoDrop (Thermo Scientific). Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (#K1672, Thermo Scientific) was used to synthesize cDNA from the samples. Primers were designed via https://eu.idtdna.com/pages/scitools and purchased from Sigma-Aldrich. The following primers were used for quantitative polymerase chain reaction (qPCR): *Agtr1a*-(NM_030985.4) forward: CATCAGTCTCCCTTTGCTATGT; reverse: AGTGACCTTGATTTCCATCTCTT.

Agtr2-(NM_012494.3) forward: CCTTCCATGTTCTGACCTTCTT; reverse: GCCAGGTCAATGACTGCTATAA. *Actin*-(NM_031144) forward:

TGTCACCAACTGGGACGATA; reverse: GGGGTGTTGAAGGTCTCAAA.

The PCR method used SYBR Green as probe. Briefly, maxima®SYBR Green qPCR Master Mix (Thermo Scientific, #K0253) was used according to manufacturer's instructions in a Hard-Shell™ 96-well PCR plate (BioRad). The reaction was conducted in duplicates using a thermal cycler (BioRad CFX96 Real-Time System) with initial denaturation at 95 °C for 10min. Denaturation and amplification were performed in 45 cycles of 95 °C for 15s, 63 °C for 30s, and 72 °C for 30s. No template control (NTC) was included in the reaction and melting curve analysis was performed. Results were analyzed using Ct values. Beta-actin mRNA levels were used for normalization of the results and $2^{-\Delta\Delta Ct}$ values were calculated for each gene.

2.9. Immunoprecipitation

Lysates from transfected MG87.TRKB/GFP-AGTR2 cells were incubated with antibody against TRK (Santa Cruz, #sc7268) overnight (1ug of Ab; 500ug of total proteins) at 4 °C. Following incubation with Protein-G Sepharose (Life Technologies, #101242) for 2 h at 4 °C, samples were centrifuged at 10000 × g for 2min, the supernatant was stored, and this cycle was repeated twice, using lysis buffer to wash the samples and the supernatant discarded, and the precipitate was stored at −80 °C until use.

2.10. Western blotting and ELISA

For western blotting, protein precipitated by anti-TRK and supernatant were separated in SDS-PAGE and transferred to PVDF membranes. Following blocking with 3% BSA in TBST, membranes were incubated with antibody against GFP (1:1000; Santa Cruz, #sc-8334) or total TRK (1:1000; Santa Cruz, #sc11-Rb). Membranes were incubated with secondary antibody conjugated to HRP (1:10000; Bio-Rad, #170–5046) and chemiluminescence emitted after addition of ECL was detected by a CCD camera. Immunoblot bands were measured using NIH ImageJ 1.32.

For ELISA, samples (120μg total proteins) were incubated overnight at 4 °C in 96-well plates previously coated with anti-TRK (1:500; Santa Cruz, #sc7268, overnight at 4 °C) and blocked with 3% BSA for 2 h at RT in PBST. Following a wash with PBST, anti-pTRK.Y816 (1:2000; Cell Signaling, #4168), biotin-conjugated anti-pY (1:2000; AbD Serotec, UK, #MCA2472B), or anti-FYN (1:2000; Santa Cruz, #sc16) was incubated overnight at 4 °C. After a wash with PBST, the plate was incubated with HRP-conjugated tertiary antibody (1:5000; Bio-Rad, #170–5046) or HRP-conjugated streptavidin (1:10000; Thermo Fisher, #21126). Chemiluminescence emitted after addition of ECL was detected by a plate reader (Varioskan Flash, Thermo-Fisher). The signal from each sample was subtracted of blank and normalized by the value of vehicle-treated samples, thus expressed as percentage of the control group.

2.11. Surface expression of TRK

Cells from rat E18 cortex were cultivated in 96-well plates as described above (DIV8). Detection of surface TRKB was performed by ELISA (Zheng et al., 2008). Cells were fixed with 4% PFA for 20 min at RT. After washing with PBS, wells were blocked with 5%

nonfat dry milk and 5% normal goat serum in PBS for 1 h at RT. Primary antibody against the extracellular portion of TRK (1:500, Santa Cruz, #sc8316) was then incubated overnight at 4 °C. Following a wash with PBS, cells were incubated with HRP-conjugated antibody (1:5000; Bio-Rad, #170–5046) for 2 h at RT. Signal detected after addition of ECL and subtracted of blank were normalized by the average of vehicle-treated samples, and expressed as percentage of the control group.

2.12. Data analysis

Statistical analyses were performed using two-tailed Student's *t*-test, one- or two-way analysis of variance (ANOVA) followed by Fisher's LSD *post-hoc* test. Criteria for statistical significance was $p < 0.05$.

3. Results

3.1. Antidepressant-like effect of losartan

Different classes of drugs with diverse mechanisms regarding RAS modulation were used to evaluate a possible drug-induced antidepressant effect. As depicted in Fig. 1c, one-way ANOVA indicates a significant effect of losartan treatment ($F_{4,46} = 8.27$, $p < 0.05$). Both losartan doses (10 and 45 mg/kg) and imipramine (30 mg/kg, used as positive control) reduced the immobility time in FST (Fisher's LSD $p < 0.05$ for all). On the other hand, captopril ($F_{3,29} = 1.83$, not significant: NS) and enalapril ($F_{3,16} = 0.46$, NS) treatment were not effective in decreasing immobility time in the FST, as found in Fig. 1a and b. The immobility time of rats exposed to a swimming session is increased after uncontrollable stress and treatment with antidepressant drugs decreased this parameter (Nestler and Hyman, 2010). Moreover, provided that known antidepressants decrease immobility time in FST, good predictive validity is attributed to this test, thus supporting FST as a screening test for putative new antidepressant drugs and their mechanisms. Since only systemic losartan exhibited an antidepressant-like effect, this drug was infused into dHC, vHC, or PL to address which of these structures may mediate such effect. The data shown in Fig. 1d–f indicate that losartan infused into vHC ($F_{3,11} = 10.66$, $p < 0.05$) and PL ($F_{3,19} = 2.72$, $p < 0.05$) but not into dHC ($F_{3,24} = 0.50$, NS), was able to decrease immobility time in the FST.

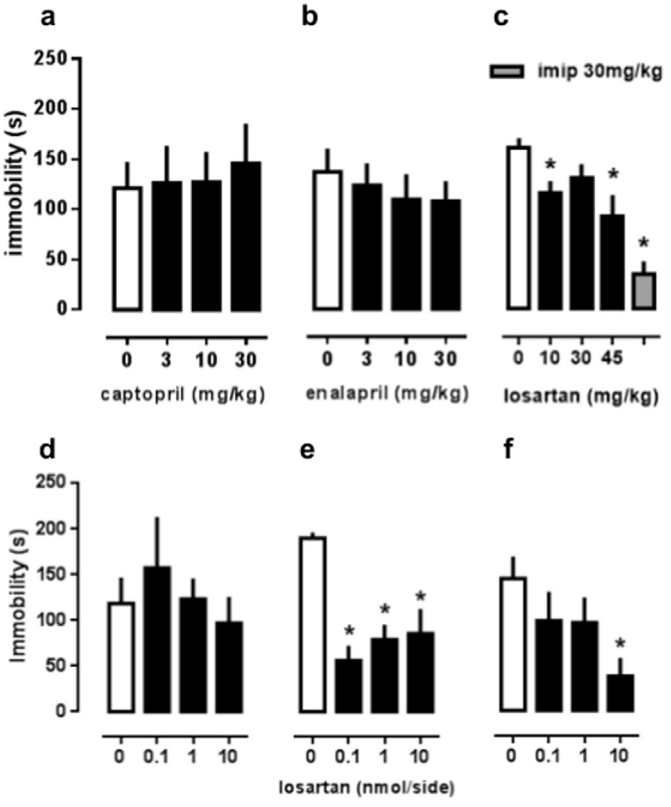

Fig. 1. Antidepressant-like effect of losartan. Animals were treated ip 24, 5, and 1 h before FST with captopril (a) at 0, 3, 10, or 30 mg/kg (n = 7–9/group) doses or with enalapril (b) at 0, 1, 3, or 10 mg/kg (n = 5/group) doses. (c) Losartan was administered ip 1 h before FST at 0, 10, 30, or 45 mg/kg and imipramine at 30 mg/kg was used as positive control (n = 5–16/group). (d–f) Losartan was bilaterally infused at 0, 0.1, 1, and 10 nmol/side into (d) dorsal hippocampus (dHC, n = 4–8/group), (e) ventral hippocampus (vHC, n = 3–4/group), or (f) prelimbic prefrontal cortex (PL, n = 5–7/group) 20min before the test session. Data are expressed as mean ± SEM of immobility time (s); **p<0.05* compared with control group.

3.2. Interaction between losartan and TRK or AGTR2: in vivo data

The requirement for TRK or AGTR2 activation (or both) for the antidepressant effect of losartan was examined. As shown in Fig. 2a and b, K252a (a TRK receptor antagonist) infused into vHC or PL was able to modulate the effect of systemically injected losartan. Two-way ANOVA revealed an interaction between factors (systemic and intracerebral injection) for both structures (vHC: $F_{1,15} = 8.62$; PL: $F_{1,13} = 6.58$; $p < 0.05$ for both). For vHC experiments, pairwise comparisons indicated that the immobility time of losartan/ctrl group is decreased compared with the ctrl/ctrl group (Fisher's LSD, $p < 0.05$). The losartan/K252a group was significantly different than the losartan/ctrl group (Fisher's LSD, $p < 0.05$), suggesting that K252a prevents the antidepressant-like effect of losartan. For PL experiments, pairwise comparisons revealed that the immobility time of animals treated with losartan/ctrl is lower than that of the control group (Fisher's LSD, $p < 0.05$). The losartan/K252a group was different compared to the losartan/ctrl group (Fisher's LSD, $p < 0.05$), suggesting that K252a also prevents losartan effect in this brain region.

We next analyzed if the antidepressant effect of losartan relies on AGTR2 activity. As shown in Fig. 2c and d, the AGTR2 antagonist PD123319 infused into PL, but not into vHC, was able to mitigate the effect of systemically injected losartan. Accordingly, two-way ANOVA revealed interactions between the compounds in PL ($F_{1,28} = 5.11$, *p<0.05*) but not in vHC ($F_{1,26} = 0.18$, NS). In addition, pairwise comparisons concerning PL experiments indicated that the immobility time of losartan/ctrl group is reduced compared with the ctrl/ctrl group (Fisher's LSD, *p<0.05*). However, the losartan/PD123319 group was not different from losartan/ctrl (Fisher's LSD: $t_{28} = 1.95$, $p = 0.06$). Therefore, activation of AGTR2 in PL, but not in vHC, is necessary to mediate the antidepressant-like effect of losartan.

Finally, we submitted BDNF haploinsufficient mice and their littermate (WT) controls to FST. As shown in Fig. 2e, the administration of losartan decreased the immobility time only in WT animals, while no effect was observed in animals with reduced BDNF levels (interaction: $F_{1,24} = 10.15$; *p<0.05*). Considering only the WT group, one-way ANOVA indicated that both losartan (45 mg/kg) and fluoxetine (30 mg/kg; used as a positive control) reduced immobility in the FST [mean immobility time in seconds/SEM/n, ctrl = 150.6/8.3/7, losartan = 64.25/15.40/8, fluoxetine = 39.8/9.65/5; $F_{2,17} = 20.44$; *p<0.05*; data not shown].

3.3. Interaction between TRK and AGTR2: in vitro data

Since the losartan antidepressant-like effect may depend mutually on TRKB and AGTR2 signaling in PL, we hypothesized that

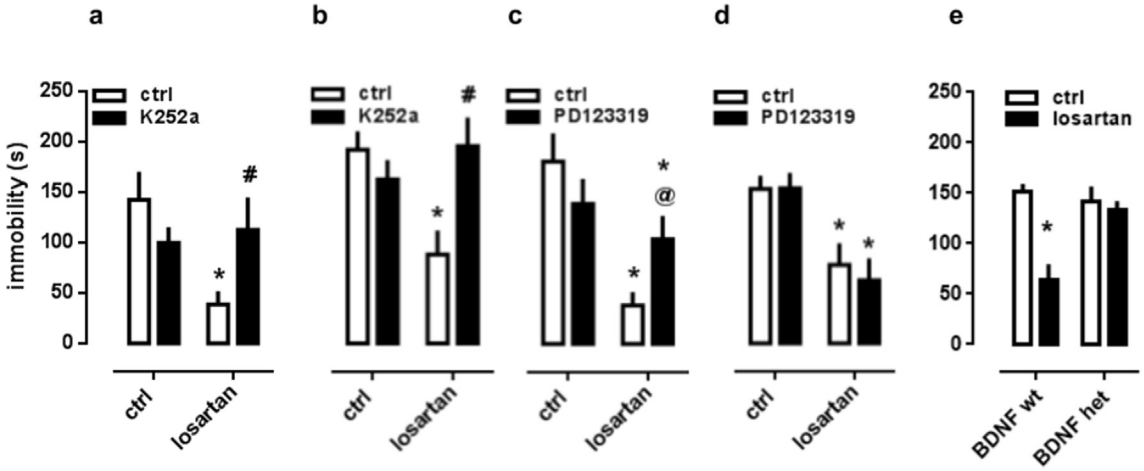

Fig. 2. *In vivo* data on interaction between losartan and TRK or AGTR2. The antidepressant-like effect of losartan was attenuated by K252a infused into (a) PL (n = 4–5/group) and (b) vHC (n = 4–6/group). The infusion of the AGTR2 antagonist PD123319 into (c) PL (n = 7–9/group) attenuated the effect of systemically injected losartan, but no change was observed in (d) vHC (n = 4–6/group). (e) Interaction between genotype and losartan effect in mice haploinsufficient to BDNF or WT littermates (n = 5–8/group). Losartan was administered 1 h before FST and either K252a or PD123319 was bilaterally infused 20 min before the test session. Data are expressed as mean ± SEM of immobility time (s). *$p<0.05$ compared with ctrl/ctrl group, unless otherwise stated; #$p<0.05$ compared with PD/VEH group. @$P = 0.06$ compared with PD/VEH group.

this drug would allow a shift of ANG2 from AGTR1 towards AGTR2 to forward TRKB activation. To test this possibility, losartan, PD123319, or K252a (first factors) was added to the primary cell cultures 10min prior to ANG2 (second factor) and pTRK levels were analyzed 10min after the last administration. Two-way ANOVA indicated an interaction between both factors ($F_{3,28} = 6.77$, $p<0.05$) and pairwise comparisons revealed that both PD123319 and K252a prevented the ANG2 effect of increasing pTRK levels (Fisher's LSD, NS for both; Fig. 3a); no additive effect was observed with the combination of ANG2 and losartan. We next determined if the ANG2 effect was dependent on BDNF release. To test this possibility, a soluble BDNF scavenger (TRKB.Fc, first factor) was added to the cell culture medium 10min before ANG2 (second factor). Two-way ANOVA did not indicate an interaction between both factors ($F_{1,20} = 0.82$, NS), suggesting that the effect of ANG2 on pTRK levels does not involve increment on BDNF release (Fig. 3b).

Next, PD123319 (first factor) was added to the culture medium 10min before BDNF (second factor) and pTRK levels were analyzed. Two-way ANOVA showed a significant interaction between factors ($F_{1,20} = 36.18$, $p<0.05$). Pairwise comparisons indicated that PD123319 abrogated the BDNF effect of increasing pTRK levels (Fisher's LSD, $p<0.05$; Fig. 3c), suggesting that AGTR2 participates in BDNF-induced TRK activation. In addition, prior administration of PD123319 (first factor) was not able to prevent the NGF (second factor) effect of increasing pTRK levels (Fig. 3d), since no interaction between both factors was observed ($F_{1,13} = 0.60$, NS). Administration of the AGTR2 agonist CGP42112 was also able to increase pTRK levels, and such effect was blocked by prior administration of PD123319 (interaction, $F_{1,20} = 9.78$; $p<0.05$; Fig. 3e).

3.4. AGTR2-dependent interaction with FYN, surface positioning of TRKB, and co-immunoprecipitation of AGTR2 and TRKB

Next, we decided to verify if ANG2 acting on AGTR2 is able to influence TRK/FYN coupling, given that FYN is a SRC member responsible for transactivation of TRK (Rajagopal and Chao, 2006). To test this, PD123319 (first factor) was added to the culture medium 10min before ANG2 or BDNF (second factors) and TRK/FYN coupling was analyzed. Interestingly, two-way ANOVA indicated a significant interaction between ANG2 and PD123319 ($F_{1,19} = 5.08$, $p < 0.05$) and, surprisingly, between BDNF and PD123319 ($F_{1,20} = 6.74$, $p<0.05$). Pairwise comparisons showed that PD123319

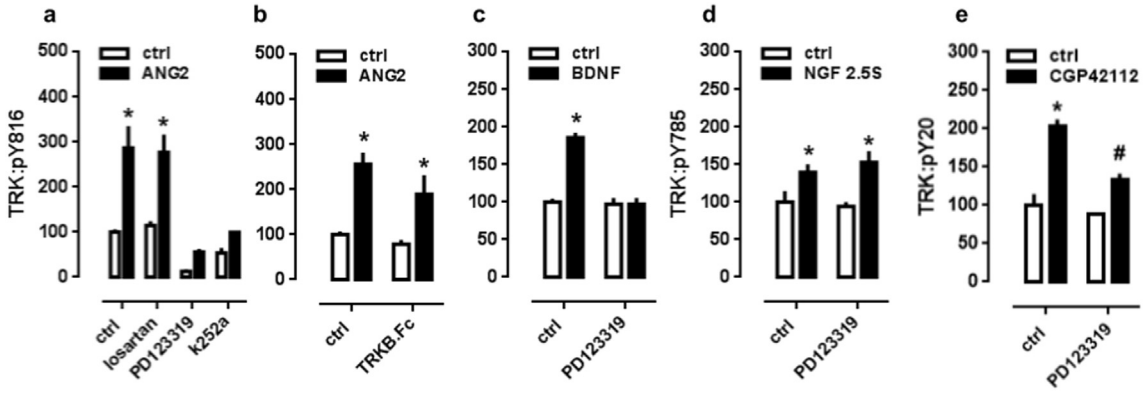

Fig. 3. *In vitro* data on interaction between TRK and AGTR2. (a) Prior administration of PD or K252a, but not losartan, blocked the ANG2-induced increase in TRKB activation in cortical cells of rat embryo (E18 cortex cells; DIV8-10, n = 4–6/group). (b) Previous incubation with TRKB.Fc did not change ANG2-induced increase in TRKB activation in cortical cells (n = 6/group). Previous incubation with PD123319 blocked (c) BDNF-induced and (e) CGP42112-induced, but not (d) NGF-induced activation of TRK (n = 4–6/group). Antagonists or TRKB.Fc were administered 10min before ANG2, BDNF or NGF. *$p<0.05$ compared with ctrl/ctrl group; cells were lysed 10 min after last drug administration.

abolished the ANG2 and BDNF effect of increasing TRK/FYN coupling (Fisher's LSD, NS for both; Fig. 4a). Next, we determined if PD123319 or ANG2 modulates surface levels of TRKB. Cultured cortical cells exposed to PD123319 had decreased surface levels of TRKB; the converse was observed with ANG2 increased ($F_{2,30} = 24.33$; Fig. 4c).

Altogether, these results point to the existence of a heterodimer TRKB/AGTR2, since previous studies have described cross-antagonism (ability of both antagonists of each receptor unit in the heterodimer to block signaling of the other's agonist) as a fingerprint of heterodimerization (Ellis et al., 2006; Ferrada et al., 2009). Consistent with this idea, GFP-tagged AGTR2 co-precipitated with TRKB (Fig. 4d).

3.5. Incubation with glutamate inverts the ratio between AGTRs in cortical cells

A preliminary comparison between the presented *in vitro* and *in vivo* analysis indicated an incompatibility regarding the effects of losartan. This compound, although effective when injected systemically or into mPFC, did not exert any effect *per se* in cultured cortical cells. Therefore, we considered the putative role of pretest stress in animals. In this scenario, a single exposure to inescapable stress, in addition to a peak in corticosterone production (lasting for 2 h), increases glutamate levels for up to 24 h (Musazzi et al., 2016; Popoli et al., 2011). Therefore, we first determined the levels of *Agtr1a* and *Agtr2* mRNA in our cultured cells. As shown in Fig. 5a, we observed a 5-fold higher expression of *Agtr2* than that of *Agtr1a* (Mean ΔCt value/SEM/n; *Agtr1a*, 17.08/0.09/3; *Agtr2*, 14.69/0.37/3; $t_4 = 2.96$, $P<0.05$). Further, we incubated cortical cells with glutamate (10 or 100uM/2 h) and determined the levels of *agtr* mRNA. A separate analysis of *agtr* expression following glutamate suggested a decrease in *Agtr2* mRNA levels (mean of fold change/SEM/n from ctrl = 1.00/0.09/5; glutamate 10uM = 0.88/0.06/5; glutamate 100uM = 0.47/0.10/4) but no change in *Agtr1a* (fold change from ctrl = 1.00/0.10/5; glutamate 10uM = 1.39/0.14/5; glutamate 100uM = 1.12/0.11/4). Under this condition, there was an inversion in the ratio between *Agtr1a* and *Agtr2* mRNA levels (glutamate 10uM = 1.5-fold more *Agtr1a*; glutamate 100uM = 2.54-fold more *Agtr1a*; Fig. 5b). Cortical cells pre-exposed to glutamate (100μM/2 h) were then tested 24 h later for the interaction between losartan and ANG2. Interestingly, 24 h after incubation with glutamate (putatively inverting the *Agtr1a/Agtr2* ratio), losartan was necessary for the effect of ANG2 on pTRK levels (interaction: $F_{1,20} = 14.46$, $p<0.05$; Fig. 5c).

4. Discussion

The present study indicates that systemic treatment with angiotensin receptor blocker (ARB) losartan and imipramine (used as positive control), but not with the ACE inhibitors captopril or enalapril, promoted an antidepressant-like effect in the FST. Losartan infusion into vHC or into PL, but not into dHC, was sufficient to mimic the antidepressant-like effect of systemic injection. Both the hippocampus and vmPFC are core structures that modulate motivational and emotional behavioral consequences of stress exposure, including depressive disorders (Castrén, 2005; Nestler et al., 2002). Consistent with our data, vHC is suggested to be mainly related to behavioral and physiological consequences of stress exposure, while dHC engages cognitive and learning processes concerning spatial navigation (Fanselow and Dong, 2010). The antidepressant-like effect of losartan probably relies on TRK signaling acting in the hippocampus and vmPFC prelimbic aspects, whereas AGTR2 activation is required only in PL, since an inhibitor of TRK in vHC and PL, and an AGTR2 antagonist in PL decreased such effect. Moreover, we can rule out any nonspecific effect on locomotion, a common misleading factor in FST, since no change was observed in this parameter following the pharmacological treatments (Fig. S1). The differences between these two structures can be explained by the higher levels of AGTR2 found in medial aspects of prefrontal cortex than in hippocampus. The study from De Kloet and colleagues (de Kloet et al., 2016), using GFP expression to report for AGTR2 expression, demonstrated positive labelling for the fluorescent protein in the medial prefrontal cortex of mice. The same study found very low levels of GFP in the hippocampus of these animals. In line with this observation, the levels of *Agtr2* in mouse hippocampus are found below the cutoff used in Gregg and cols. study (Gregg et al., 2010). However, using a functional approach it was observed that ANG2 amnesic effect is counteracted by the AGTR2 antagonist PD123319 in the hippocampus (Kerr et al., 2005).

The sequential behavioral approach used in the present study reinforced the involvement of the BDNF/TRKB system in the effect of losartan. In mice with dampened BDNF expression, losartan was no longer able to exert antidepressant-like effects. Similar to what was observed after losartan treatment, these mice are not responsive to classical antidepressants, such as imipramine and fluoxetine (Castrén and Antila, 2017; Karpova et al., 2011; Saarelainen et al., 2003).

To corroborate the behavioral experiments and to further explore the mechanisms involved in losartan effects, we used

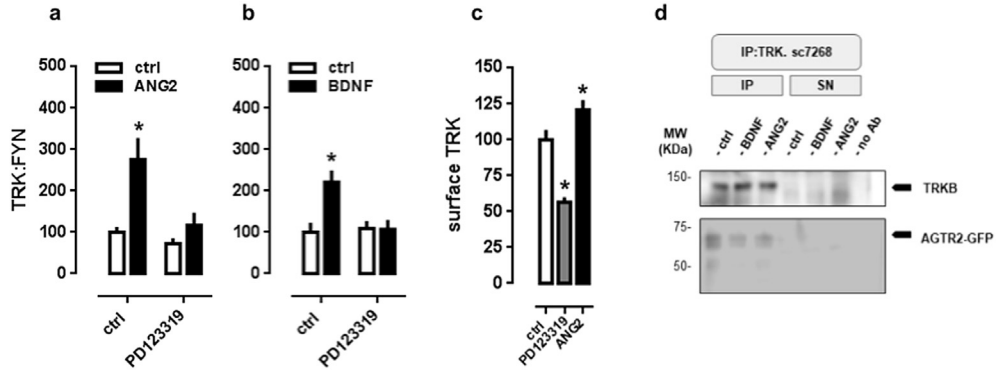

Fig. 4. AGTR2-dependent interaction with FYN, surface positioning of TRKB, and co-immunoprecipitation of AGTR2 and TRKB. Prior treatment with PD123319 impaired TRK:FYN coupling induced by (a) ANG2 (n = 5–6/group) or by (b) BDNF (n = 6/group). (c) PD123319 decreased while ANG2 increased the levels of TRKB on the surface of cultured cortical cells (n = 9–12/group). (d) Sample from MG87.TRKB fibroblast cell line, overexpressing AGTR2 labeling on blotting membrane from immunoprecipitation of TRKB protein. *$p<0.05$ compared with ctrl/ctrl group, unless otherwise stated; cells were lysed 10 min after last drug administration.

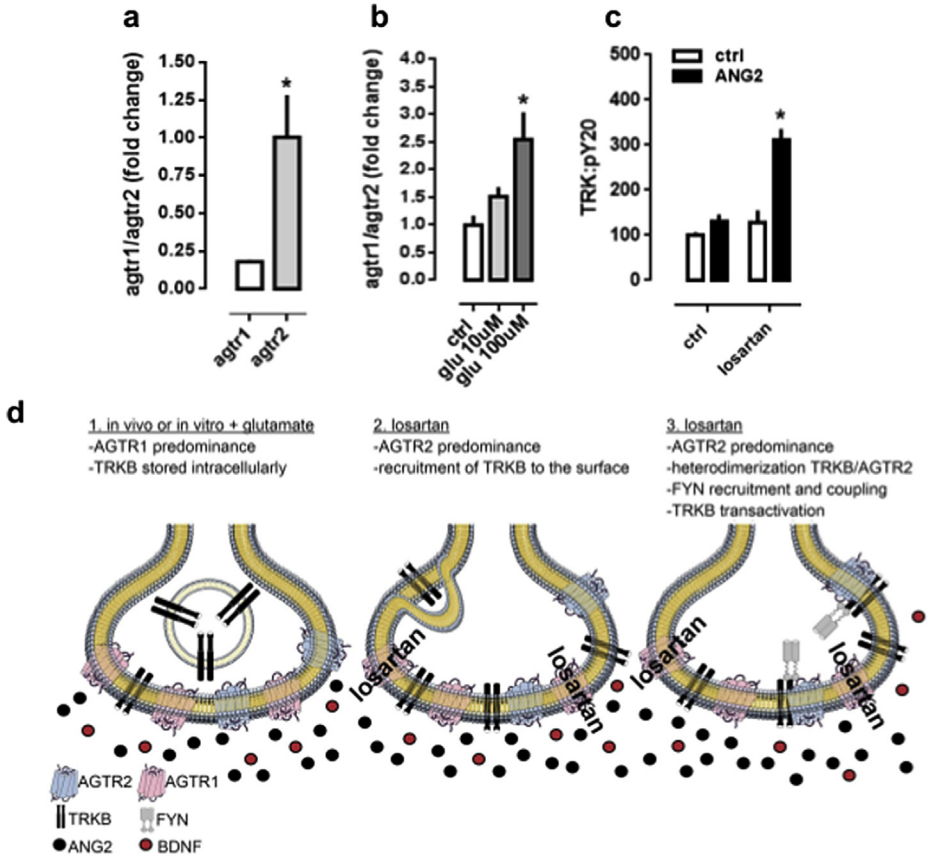

Fig. 5. (a) *Agtr2* subtype has a 5-fold higher mRNA expression than *Agtr1a* (n = 3/group). (b) Incubation with glutamate (10 or 100 μM/2 h) inverts this ratio in favor of AGTR1. (c) Cells challenged with glutamate (100 μM/2 h) and tested for ANG2-induced activation of TRKB 24 h later were responsive only in the presence of losartan (n = 6/group). *$p<0.05$ from ctrl or *Agtr1a* group. (d) Graphical abstract for the TRKB-dependent antidepressant-like effect of losartan. *In vivo* basal conditions, the high levels of AGTR1 compared with AGTR2 in prefrontal cortex would be responsible to maintain TRKB out of the cell surface. Upon blockade of AGTR1, the activation of AGTR2 increases surface levels of TRKB and favors its docking to FYN.

primary cultures of embryonic cortex to evaluate the interaction between RAS and BDNF/TRKB signaling. First, we observed that K252a and PD123319, but not losartan, prevented the ANG2 effect of increasing pTRK levels, thus suggesting a putative action on AGTR2. This possibility is strengthened by the administration of AGTR2 agonist CGP42112, that also induced an increase in TRK activation, and this effect was counterbalanced by prior incubation with the AGTR2 antagonist PD123319. Since the soluble form of TRKB (TRKB.Fc) did not prevent the ANG2 effect on pTRK, it is plausible to consider that AGTR2-induced activation of TRKB would not require increment in BDNF release. In this sense, transactivation of TRKB or a facilitatory effect of basal levels of BDNF on its receptor are reasonable scenarios. Previous studies have described that both the GPCR ligands adenosine and pituitary adenylate cyclase-activating polypeptide can transactivate TRK (Rajagopal et al., 2004). In addition, TRK transactivation by an adenosine 2A receptor agonist was blocked by PP1, suggesting involvement of the SRC family tyrosine kinase (Lee and Chao, 2001). Thus, FYN and other SRC-family kinases are responsible for TRK transactivation (Huang and McNamara, 2010; Rajagopal and Chao, 2006), and lipid raft localization of TRKB is regulated by FYN (Pereira and Chao, 2007). Consistent with this evidence, we observed that ANG2 was also able to increase levels of TRK/FYN coupling in cortical cultures. Therefore, we propose that FYN acts as an intermediary molecule capable of inducing TRKB transactivation when ANG2 acts on AGTR2. Moreover, BDNF itself promotes TRKB/FYN coupling (Iwasaki et al., 1998). Corroborating that prospect, our data also showed that BDNF increased TRKB/FYN coupling. Therefore, both ANG2 and BDNF, which are able to increase pTRK levels, also induce TRK/FYN coupling.

As expected, PD123319 blocked TRKB/FYN coupling from ANG2 action, but unexpectedly PD123319 also prevented such coupling from BDNF action. Also unexpected was the data indicating that PD123319 prevents the BDNF effect of increasing pTRKB levels. A generalized interaction of AGTR2 with other TRK members is unlikely, as PD123319 did not prevent NFG action of increasing pTRKA levels. These unforeseen interactions can be explained by the observation that PD123319 was able to reduce surface expression of TRKB, whereas ANG2 led to an increase, thereby suggesting a putative displacement of TRK to the surface upon AGTR2 signaling and a decrease of BDNF effectiveness with prior PD123319. Indeed, modulation of TRK surface trafficking is important considering the two possible scenarios mentioned above for the activation of TRKB, which may occur on cell membrane (Rajagopal et al., 2004). In addition, the MG87.TRKB cell line, which overexpresses TRKB, allowed us to observe co-immunoprecipitation of GFP-tagged AGTR2 and TRKB, suggesting AGTR2/TRKB dimerization. This approach was chosen for the following two reasons: first, as analyzed by the group of Juan Saavedra, commercially available antibodies against AGTRs are far from ideal (Hafko et al., 2013); and second, the cell line used expresses exclusively TRKB, thus being an ideal tool for our purpose.

A preliminary analysis showed that *Agtr2* mRNA levels were 5-fold higher than *Agtr1a* in our primary cultures, and this ratio

was inverted to 2.5-fold more *Agtr1a* after incubation with glutamate. This later feature seems to allow a cooperative effect of losartan and ANG2. Using a model of retinal ischemia, it was observed that increased expression of *Agtr1a* mRNA peaked 12 h after reperfusion, while treatment with candesartan was able to prevent ischemia-induced glutamate release (Fujita et al., 2012). Taken together, these data indicate a possible positive feedback between AGTR1 signaling and glutamatergic transmission. Moreover, consistent with our *in vitro* observations, *Agtr1a* levels increased while *Agtr2* levels decreased in the medulla of stress-induced hypertensive rats (Du et al., 2013). However, an opposite effect of glutamate on *Agtr2* mRNA has also been described (Makino et al., 1998). In this study, glutamate insult led to an increase of *Agtr2* mRNA. The precise mechanism where glutamate release might reduce the levels of *Agtr2* are still not understood and these apparent discrepancies could rest on methodological differences. For example, the culture method of Makino and colleagues rely on cortical cells cultivated for 14 days, supplemented with calf serum and mitosis inhibitors; our cultures were serum-free (substituted by B27) and cultivated for 8 days without any drugs to prevent cell proliferation.

Given that the FST protocol for mice does not engage the exposure to a previous stressful session, posits that *Agtr1a* levels could be kept higher by basal glutamatergic transmission rather than by stressful stimuli as postulated before. To address this possibility, we used the EMBL-EBI Expression Atlas (https://www.ebi.ac.uk/gxa/home) to compare the expression of *Agtr1a* and *Agtr2* in the murine brain. We only obtained data from RNAseq experiments conducted in four strains of mice by Gregg and colleagues (Gregg et al., 2010). Consistent with the proposed scenario, these authors observed that *Agtr2* levels were lower or at least equivalent to *Agtr1a* in the medial prefrontal cortex of adult mice. Our behavioral data, indicating the effectiveness of losartan in mice submitted to FST, also suggests that the *Agtr1a/2* ratio *in vivo* favors *Agtr1a*, since no previous stressful event is presented in this protocol.

The data obtained from mouse FST, indicating that animals lacking one copy of BDNF gene do not respond to losartan, is also apparently contradicting the *in vitro* lack of interaction between TRKB.Fc and ANG2. However, the differences between the systems used can be crucial to the observed responses. As mentioned, in adult prefrontal cortex, the levels of *Agtr1a* are higher than *Agtr2*, while we observed the opposite in the cultivated cells. Another source of variation relies on the fact that cultivated cells are not part of an intrincated, fine-tuned network, therefore submitted to less influence of the neighboring cells or structures. Supporting our *in vivo* findings, the decrease of BDNF levels in rats through the injection of shRNA, blocked the neuroprotective effects of candesartan in a model of stroke (Fouda et al., 2017).

The activation of AGTR1 increases BDNF expression, as found in human and rat adrenocortical cells (Szekeres et al., 2010), an effect shared by AGTR2 (Alhusban et al., 2013; Namsolleck et al., 2013), and associated to the improvement in cognitive performance after telmisartan (Kishi et al., 2012). Thus, in a speculative scenario, there is a positive feedback on BDNF production by both receptors. However, the AGTR2/TRKB heterodimer is positioned in the membrane; given that the majority of TRKB is internalized (Haapasalo et al., 2002); where it can be activated by BDNF. Such effect of BDNF would then be enhanced by the recruitment of FYN or other SRC-family kinases by the activation of AGTR2.

In conclusion, our findings indicate that losartan induces an antidepressant-like effect possibly mediated by AGTR2 and TRKB coupling in the mPFC. We observed a previously unknown TRKB activation by AGTR2, involving recruitment of the SRC family kinase FYN. Our results suggest that TRKB/AGTR2 heterodimer docks the FYN kinase to promote a crosstalk that putatively induces pTRKB/PLCγ1 signaling. However, the higher levels of AGTR1 in the prefrontal cortex prevents the ANG2-triggered docking of FYN to the AGTR2/TRKB complex. Losartan, by blocking AGTR1, facilitates the AGTR2-mediated effects of ANG2 on TRKB. Considering the high comorbidity between depression and cardiovascular disorders, our results suggest that ARBs, such as losartan, could be a therapeutically viable option in these cases or they could even be a putative novel class of antidepressant drugs. Still, the present and other studies allow us to conclude that the relationship between RAS and BDNF/TRKB systems are far from straightforward, and further studies are necessary, taking into account the limitations of each approach used.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.03.011.

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